



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

ZHANG and PURCHIO

Serial No.: 09/465,978

Art Unit: 1632

Filing Date: December 16, 1999

Examiner: R. Shukla

Title: **METHODS AND COMPOSITIONS FOR SCREENING FOR ANGIOGENESIS
MODULATING COMPOUNDS**

DECLARATION UNDER 37 C.F.R./1.132

I, Ning Zhang, declare as follows:

1. I am presently a Senior Research Scientist at Xenogen, Corporation and have held this position since 1999. Prior to joining Xenogen, I was a post-doctoral fellow at Tularik, Inc in South San Francisco, CA. I received my PhD in microbial molecular genetics from the University of Adelaide, Australia in 1993 and hold a Bachelor of Science degree from Lai Young Agricultural University in China. A copy of my Curriculum Vitae (Exhibit A) is attached hereto. I have considerable experience in the area of molecular biology and in the development of transgenic animal models for studying angiogenesis and inflammation-mediated diseases.

2. I am a co-inventor of the above-referenced application (hereinafter "the specification") and am familiar with the contents of the specification. I also understand that the claims as pending are directed to a transgenic mouse into which an expression cassette is introduced. The expression cassette includes a VEGFR-2 regulatory sequence set forth as SEQ ID NO:32 in the application operably linked to a sequence encoding a light-generating protein, for example luciferase.

3. We have demonstrated that the VEGFR-2 promoter sequence set forth as SEQ ID NO:32 in the specification directs expression of a light-generating protein (such as

*considered
R/S
2/24/02*

luciferase) in transgenic mice. Additionally, we have demonstrated that luciferase activity in these transgenic mice actively mimics the change in angiogenesis activity. Thus, the results from these studies show that transgenic mice as claimed can be utilized to screen compounds for their ability to regulate expression of angiogenesis via VEGFR-2 regulatory regions.

4. The VEGFR-2 promoter sequence used to generate transgenic mice was prepared as described in Example 3 of the specification (page 65, line 24 to page 66, line 7). Briefly, a 4.6 kb *HindIII*-*XbaI* fragment that covers the VEGFR-2 promoter region was subcloned from the VEGFR-2 BAC clone into vector pSK (Stratagene, La Jolla, CA) linearized with *HindIII* and *XbaI*. This construct was designated pSK-K6. Restriction analysis of the construct confirmed that the 4.6 kb promoter region was cloned. The pSK-K6 construct was then re-engineered to delete a 159 bp sequence from the ATG translational start codon to a downstream *XbaI* site. A 0.3 kb 3' end fragment was obtained by PCR amplification of pSK-K6 using the forward primer VR2F (CGC TAG TGT GTA GCG GCG GCT CTC; SEQ ID NO:30 of the specification) and the reverse primer VR2R (ATA AGA ATG CCG CCG GCT GCA CCT CCG GCT GGG CAC AG; SEQ ID NO:31 of the specification). (See, also page 65, lines 28-29 of the specification). This PCR product was digested with *Bsu36I* and *NotI*, and the resulting fragment was then used to replace the 0.45 kb *Bsu36I*-*NotI* fragment of pSK-K6. The resulting construct, designated pSK-KP (page 66, line 1 of the specification), contains a novel 4.5kb VEGFR-2 promoter sequence beginning at a *HindIII* site 5' to the ATG translational start codon, and ending at that ATG codon. This promoter fragment was fully sequenced; and the sequence is shown SEQ ID NO:32 of the specification.

5. A VEGFR-2 enhancer sequence was prepared as described in Example 3 of the specification. In brief, the enhancer sequence was amplified by PCR from the VEGFR-2 BAC clone DNA using the forward primer VEF (ACA CGC CTC GAG AAA TGT GCT GTC TTT AGA AGC CAC TG; SEQ ID NO:33 of the specification) and the reverse primer VER (ACA CGC GTC GAC GAT CCA ATA GGA AAG CCC TTC CAT AAA C; SEQ ID NO:34 of the specification). The 511 bp VEGFR-2 enhancer sequence was digested with *XhoI* and *Sall* and then cloned into the *Sall* site of the pSK vector. The resulting construct was designated pSK-KN.

6. Subsequently, a VEGFR-2 promoter-luciferase-VEGFR-2 enhancer expression cassette as shown in Figure 12 of the specification was constructed. As described on page 67 of the specification, this expression cassette was constructed by isolating the VEGFR-2 promoter of SEQ ID NO:32 pSK-KP (see, paragraph 3 above) and a *HindIII*-*NotI* fragment and cloned into the same sites of pGL3B2 to generate a construct was designated pGL3B2-KP. Next, the VEGFR-2 enhancer was isolated from pSK-KN as a *XhoI*-*Sall* fragment and cloned into the pGL3B2-KP vector that had been linearized with *Sall*. The resulting construct was designated pGL3B2-KPN.

7. The transgenic vector pGL3B2-KPN was then used to generate VEGFR2-luciferase transgenic mice by DNA microinjection into the nuclei of embryonic cells derived from FVB donor. Such methods are well-known and described, for example, in Example 4 of the specification. (See, also, page 50, line 27; page 53, line 16 to page 54, line 15; page 55, line 27 to page 56, line 9; and page 59, line 28 to page 60, line 9 of the specification for discussions and references relating to transgenic mice). Using standard DNA microinjection method, 37 pups were obtained and PCR analysis showed that 8 of them were positive for the transgene. Each positive mouse was used as a founder for breeding of a transgenic colony. Genotypic analysis of the four founder lines (designated KA, KC, KG and KJ) was performed and all the founder lines were found to have different genotypes. Thus, the VEGFR2-luc-transgene has multiple chromosomal locations in these founder lines. (See, Exhibit B, Figure 1).

8. It is known that VEGFR-2 is expressed during angiogenesis and is regarded as an angiogenesis marker. (See, e.g., page 24, line 21 to page 22, line 21 of the specification). In addition, it is known that the process of angiogenesis is very active during embryonic development and in the early stages of post-natal development. Accordingly, the transgenic mice containing SEQ ID NO:32 operably linked to a sequence encoding light-generating protein (e.g., luciferase) were evaluated for luciferase expression *in vivo* from 1 week until 6 weeks of age, essentially using methods described on page 24, lines 19-21 and page 45, lines 8-24 of the specification. All the four founder lines expressed high level of luciferase activity in the entire body when 1-week-old mice were imaged. The activities declined rapidly (by up to 7000 fold) over time and by 6 week after birth, the luciferase activity dropped to a basal level (Exhibit B, Figure 2a).

These results demonstrate that VEGFR-2 expression can be monitored in transgenic mice containing a sequence encoding a light-generating protein which is operably linked to the regulatory sequence of SEQ ID NO:32 and that development-dependent reduction of luciferase activity mimicked the change of angiogenesis activity during post-natal development. Thus, transgenic mice prepared according to the teachings of the specification can be used to monitor angiogenesis *in vivo* and can be used to screen the effect of a compound on angiogenesis.

9. In light of these results, I conclude that the methods described in the specification teach how to generate transgenic mice in which expression of a light-generating protein is driven by the sequence set forth as SEQ ID NO:32 of the specification and that these animals can be used to monitor angiogenesis. Indeed, using approaches described in the specification, one can readily generate transgenic mice as set forth in the claims and use these mice to screen compounds that modulate VEGFR-2-mediated angiogenesis, including diseases and processes associated with VEGFR-2-mediated angiogenesis.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 1-4-02

Signature: 

Ning Zhang, PhD.